Journal of Chromatography, 427 (1988) 239–246
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 4141

CHROMATOGRAPHIC ANALYSIS OF THE TRINITROPHENYL DERIVATIVES OF INSULIN

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(First received November 16th, 1987; revised manuscript received January 27th, 1988)

SUMMARY

The insulin molecule was derivatised by reaction with trinitrobenzenesulphonic acid (TNBS), which is known to react predominantly with free primary amino groups. The products of the reaction were analysed by reversed-phase chromatography and by further derivatisation with dansyl chloride. Under the conditions of these experiments, TNBS was found to react preferentially with glycine at position A1. This finding is discussed in terms of the tertiary structure and immunogenicity of this derivative.

INTRODUCTION

The covalent modification of proteins by the addition of dinitrophenyl (DNP) and trinitrophenyl (TNP) groups has been extensively used in the study of immunological responses. The interaction of these groups with antibody is largely independent of where on the protein backbone they are situated (provided they are freely accessible), or to which protein they are coupled. In contrast, stimulation of T lymphocytes by these antigenic groups depends critically on the local environment of the substituent group in the protein molecule [1]. The reasons for this difference are complex, but probably result from the tripartite interaction

0378-4347/88/$03.50

between receptor, antigen and major histocompatability complex molecule which is an essential feature of T cell activation [2].

We have been interested in studying the details of T cell recognition using, as a model, trinitrophenylated derivatives of insulin. An essential requirement for this study was the identification of the specific sites of attachment of the TNP group to the insulin molecule. Although a number of functional groups in proteins can be derivatised with nitrophenyl groups, the use of the reagent 1,3,5-trinitrobenzenesulphonic acid (TNBS) has been shown to restrict modification to primary amino groups [3]. Insulin has three amino groups which could be derivatised in this way, the α-amino groups at the N termini of the A and B chains, and the ϵ-amino group on B29 lysine. A previous study on reaction of insulin with 2,4-dinitrobenzenesulphonic acid (DNBS) at pH 11.5 results mainly in a single monosubstituted product, in which the DNP molecule is attached to lysine B29 [4].

This paper describes a reinvestigation of the problem of derivatisation of insulin with nitrobenzenesulphonic acids. We report a study on the reaction of insulin with TNBS at pH 8.2. The insulin was labelled with dansyl chloride following reaction with TNBS. Doubly derivatised insulin was hydrolysed in 6 *M* hydrochloric acid and dansylated amino acids, representing amino groups not derivatised by TNBS, were identified by reversed-phase high-performance liquid chromatography (HPLC).

Using these methods we have been able to show that, in our conditions, the TNP group can be introduced predominantly to the α-amino group of A1 glycine.

EXPERIMENTAL

Reagents

Beef insulin was a kind gift from Boots. Dansyl chloride, dansyl amino acid standards and TNBS were purchased from Sigma (St. Louis, MO, U.S.A.). TNP-lysine was purchased from Biosearch (San Raphael, CA, U.S.A.). TNP-glycine was synthesised as described below. Water, acetonitrile (both HPLC grade) and trifluoroacetic acid were obtained from F.S.A. (Loughborough, U.K.). Acetone, AR grade, was purchased from May and Baker (Dagenham, U.K.).

Preparation of TNP-insulin

TNBS (70 mg) was dissolved in 1 ml of 0.1 *M* sodium bicarbonate (pH 8.2) just before use, and an aliquot was added slowly to insulin (10 mg/ml) in 0.1 *M* sodium bicarbonate (pH 8.2). A 140-μg amount of TNBS was added per mg insulin, to give a final molar ratio of 3:1. The reaction was allowed to proceed for 2 h at room temperature in the dark, although preliminary experiments suggested that the reaction was essentially complete in a much shorter time period. TNP-insulin was separated from unreacted TNBS and small-molecular-mass by-products on a Sephadex G25 (Pharmacia) column (15 cm$\times$6 cm), pre-equilibrated in 0.1 *M* sodium bicarbonate (pH 8.2). The major coloured band was collected and lyophilised.

Dansylation

Samples were reconstituted in a small volume of 0.1 *M* sodium bicarbonate (final concentration approximately 5 mg/ml) and 0.1 ml of dansyl chloride solution (2.5 μg/ml) was added. The reaction was carried out at room temperature, for 1 h in the dark. The solvent was evaporated (Savant rotary evaporator) and then hydrolysed.

Hydrolysis

Peptides were hydrolysed in 6 *M* hydrochloric acid for 16 h at 110 °C, in sealed evacuated hydrolysis tubes (Sterilin, Teddington, U.K.). Hydrolysates were dried in a rotary evaporator and stored at −20 °C until required.

High-performance liquid chromatography

Chromatography was carried out on a Varian 5000 liquid chromatograph unit, with a C_{18} μBondapak reversed-phase cartridge in a Z module holder (Waters, Milford, MA, U.S.A.). Absorbance was measured at 280 nm. For analysis of insulin and its TNP analogues, the aqueous phase used was 0.1 *M* phosphate buffer (pH 3.6). For separation of trinitrophenylated and dansylated amino acids, the aqueous phase was 0.1% trifluoroacetic acid. Acetonitrile was used as the organic modifier in all cases. Details of gradients and flow-rates are given for each separation in the Results section.

Synthesis of TNP-glycine

TNP-glycine was synthesised by reacting equimolar amounts of TNBS and glycine in 0.1 *M* sodium bicarbonate (pH 8.2). No attempt was made to purify the TNP-glycine from any unreacted glycine or TNBS derivatives.

Spectrophotometric analysis of TNP-insulin

The absorbance of a sample of TNP-insulin was measured at 280 and 340 nm in a Pye-Unicam SP8-400 spectrophotometer. The concentration of TNP-insulin and the number of moles of TNP reacting with each mole of insulin was calculated, using published values for the molar absorptivity of insulin at 280 nm and TNP-amino acids at 280 and 340 nm, and considering the absorbance of insulin at 340 nm as negligible. Using this method coupling ratios (TNF/insulin) obtained were 1:1 ±20% [5].

RESULTS

TNP-insulin consists predominantly of one species

Although spectrophotometric analysis suggested that insulin was modified with only one TNP group per molecule, this analysis is subject to a number of possible sources of error [6]. Furthermore, the result obtained gives no indication of whether the reaction occurs predominantly at one site, or at different sites on different individual insulin molecules. In order to analyse the composition of the TNP-insulin preparations, samples were analysed by reversed-phase HPLC, using a 0.1 *M* sodium phosphate (pH 3.6)–acetonitrile gradient elution system as

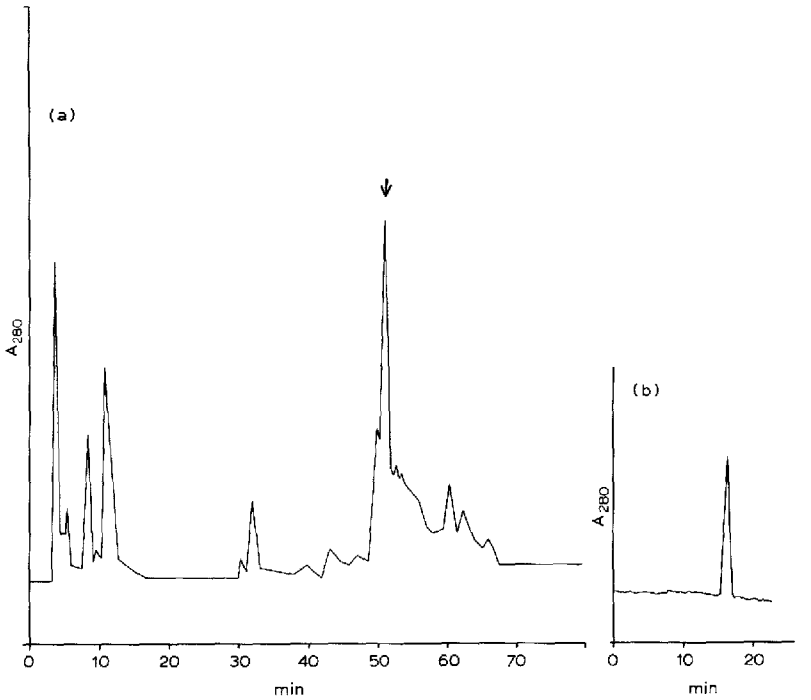

Fig. 1. (a) Preparative separation of insulin derivatives produced by reaction with TNBS. TNP-insulin was made as described in Experimental and separated by reversed-phase chromatography on a C_{18} column. Aqueous solvent: sodium phosphate (0.1 *M*, pH 3.6); organic solvent: acetonitrile. Acetonitrile concentration was maintained at 25% for 10 min, and then increased linearly to 45% over a further 60 min. Flow-rate: 1 ml/min. Arrow indicates peak collected for further analysis. (b) Analysis of major peak collected as shown in (a). A sample of the major peak collected as described above was rechromatographed using isocratic elution with 36% acetonitrile.

shown in Fig. 1. This analysis, which was very consistent from one preparation of TNP-insulin to another, showed that, at least under the conditions of this separation, the TNP-insulin consisted predominantly of a single component. Furthermore, if the samples corresponding to the major peak were collected and chromatographed under isocratic conditions (Fig. 1b), only one component could be detected. No free insulin could be detected in these preparations. Our interpretation of these results is that TNP-insulin consists predominantly of a single haptenated form. The other small peaks on the chromatogram may correspond to other mono- or dihaptenated products, but were not analysed further.

TNP reacts predominantly with A1 glycine

The three dansylated amino acids of interest to this study (glycine A1, lysine B29 and phenylalanine B1) were all found in acid hydrolysates of insulin reacted with dansyl chloride, and could be readily separated from each other in approximately equal amounts by isocratic elution in 0.1% trifluoroacetic acid–acetonitrile (Fig. 2a and b). Although the hydrolysates gave a rather complex elution pattern, because dansyl groups can modify a number of other amino acids in the insulin

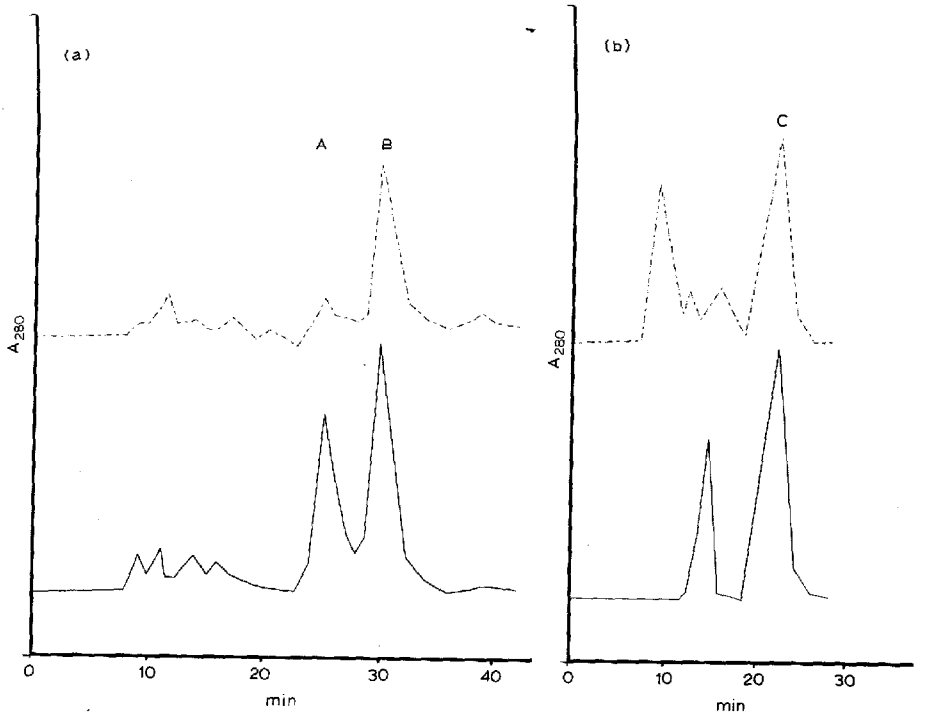

Fig. 2. Analysis of free amino groups in insulin (solid line) and TNP-insulin (dashed line) by dansylation and hydrolysis as described in Experimental. Dansyl amino acids were separated by reversed-phase chromatography and identified by comparison to dansyl amino acid standards. Aqueous solvent: 0.1% trifluoroacetic acid; organic solvent: acetonitrile. Isocratic elution using (a) 5% acetonitrile and (b) 20% acetonitrile. Dansyl standards: (A) glycine; (B) lysine; (C) phenylalanine.

molecule, the relevant amino acids were readily identified by comparison to the respective commercially available dansyl amino acid standards. However, as shown in Fig. 2a and b, identical dansylation of TNP-insulin showed an almost total absence of dansyl glycine, suggesting that this amino acid had been protected from dansylation by prior modification with TNP.

In order to confirm that the major component of TNP-insulin identified by reversed-phase chromatography was indeed haptenated at the A1 glycine position, a preparation of TNP-insulin was first fractionated according to the protocol shown in Fig. 1. The samples corresponding to the major peak were collected, pooled and then subjected to the same analysis as described above for the unfractionated mixture. As shown in Fig. 3a and b, dansylation and hydrolysis of this material revealed that both B1 phenylalanine and B29 lysine were available for reaction with the dansyl group, but no dansyl glycine should be detected. The agreement between the results obtained on analysis of unseparated TNP-insulin and the major peak obtained after chromatography confirm that TNBS reacts predominantly with A1 glycine.

In order to confirm directly the results of this study, an acid hydrolysate of TNP-insulin was analysed for the presence of TNP-glycine, by isocratic elution in a 22% trifluoroacetic acid–acetonitrile mixture. This analysis was greatly sim-

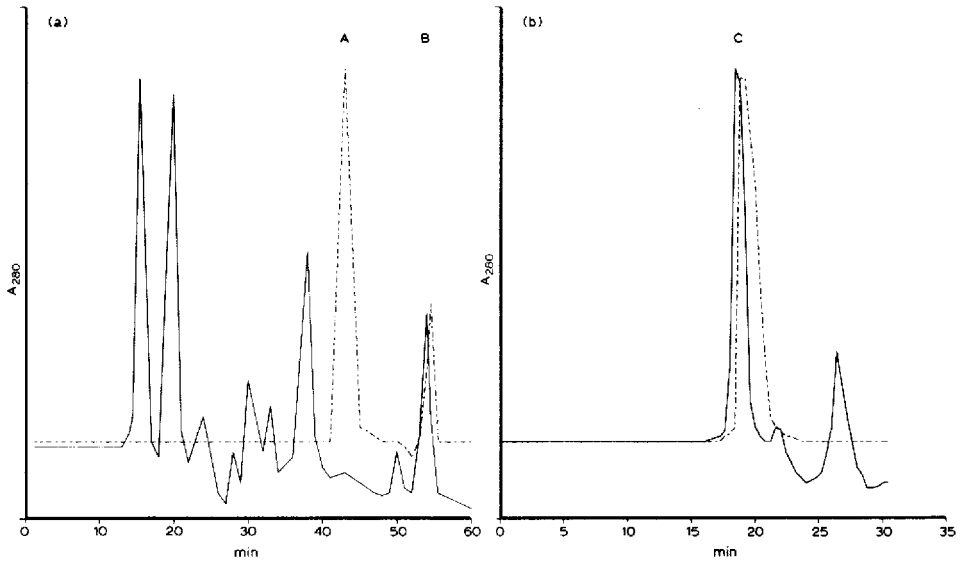

Fig. 3. Analysis of free amino groups in purified TNP-insulin. The major peak identified in Fig. 1 was collected, dansylated and hydrolysed. Dansyl amino acids were separated as in Fig. 2 and identified by comparison to dansyl amino acid standards. (a) 8% acetonitrile; (b) 26% acetonitrile. (——) Hydrolysed TNP-insulin; (----) dansyl standards. (A) Glycine; (B) lysine; (C) phenylalanine.

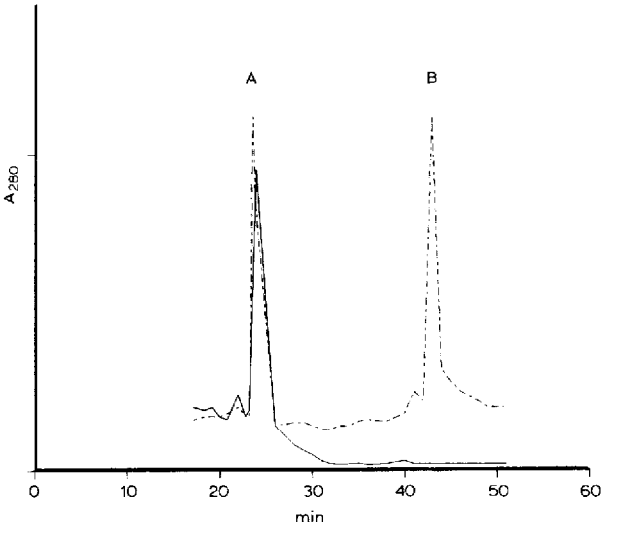

Fig. 4. Direct identification of TNP amino acids in TNP-insulin. TNP-insulin was hydrolysed, and the amino acids were separated as in Fig. 2. The elution profile of TNP-glycine and TNP-lysine standards is shown for comparison. Chromatography was carried out using the same solvents as in Fig. 2, with 22% acetonitrile. (——) Hydrolysed TNP-insulin; (----) TNP standards. (A) Glycine; (B) lysine.

plified by the fact that TNP groups adsorb at 280 nm, unlike most free amino acids. The TNP amino acids were identified by comparison to ϵ-TNP-lysine and

TNP-glycine. As shown in Fig. 4, the results unequivocally identified the presence of TNP-glycine, but not lysine in the hydrolysate.

DISCUSSION

The results of this study show that, in the reaction conditions used in the present study, TNBS reacts predominantly with the α-amino group of A1 glycine.

The only other published studies of nitrophenyl derivatives of insulin are those of Li [4] and Keck et al. [7,8], who both follow the same protocol. Under the reaction conditions used in these studies (pH 11.5, 24 h) DNBS reacts predominantly with the B29 lysine amino group. We have not followed this protocol since the insulin molecule may undergo structural modifications at these high pH values.

However, a number of others studies have shown that the three free amino groups of the insulin molecule can be derivatised to a different extent, and that the order of preference can change under different reaction conditions. For example, modification of insulin using hydroxysuccinimide esters has been shown to result primarily in A1-substituted derivatives when the reaction is carried out at pH 8.5 whilst at pH 6.9, the A1 and B1 α-amino groups are derivatised in approximately equal quantities [9]. In both cases, derivatisation of the chain termini is favoured over derivatisation of the B29 side-chain. The reasons for side-chain preference are incompletely understood, but probably involve the pK values of the different amino groups and the structural accessibility of the side-chains [10]. The discrepancy between our results and those of the earlier studies may therefore be due to some differences in the reaction conditions used.

It is important to consider the possible effect of the TNP substitution on the structure of the insulin molecule. Insulin is a small globular protein of relative molecular mass 5700 which aggregates to dimers and hexamers [11]. It consists of two polypeptide chains, designated A and B, connected by two inter-chain

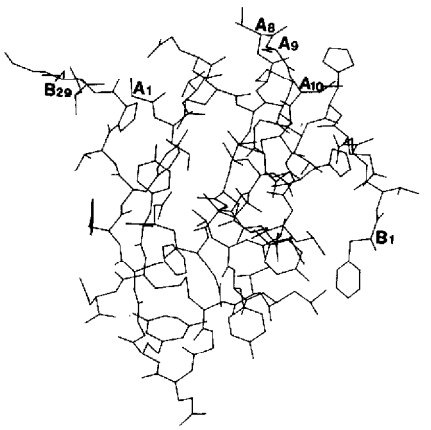

Fig. 5. Porcine insulin monomer showing residues with free amino groups (A1, B1, B29) and residues A8, A9 and A10 which are important in determining T cell recognition (porcine insulin coordinates from Brookhaven Databank).

disulphide bonds (Fig. 5). The A chain consists of two helical regions A2 to A8 and A13 to A20, connected by a more extended region of polypeptide chain. An intra-chain disulphide bond formed between the cysteines at A6 and A11 creates a loop containing the region A8 to A10 which is believed to be important in determining the T cell recognition of insulin in some mice strains. The B chain has an extended region from B1 to B8, an α-helix from B9 to B19, a turn from B20 to B23 and an extended region from B24 to B30 which forms an antiparallel β-sheet with the corresponding region of the other monomer in dimer formation. The A1 residue lies in a surface pocket close to the dimer-forming surface.

Analogues with substituent groups on the A1 amino-group have been studied extensively to determine their biological, spectroscopic and structural properties [12,13]. As well as affecting the ability of insulin to form dimers, addition of bulky groups to this region of the molecule has been shown to affect the three-dimensional structure of the insulin monomer, affecting particularly the helical region A2 to A8. Comparison of the different monomer conformations found in different crystal forms has shown that changes affecting this helix are associated with relative movements of other regions of the molecule [14], including conformations of the A8 to A10 region which may be associated with T cell recognition. Studies using computer modelling of the modified insulin molecule are in progress to try and predict these changes and to relate them to changes in immunological recognition.

ACKNOWLEDGEMENTS

G.R.W. is supported by an MRC studentship. B.M.C. is the recipient of a Royal Society University Research Fellowship. This work was supported in part by grants from the Wellcome Trust and the British Diabetic Association. We are very grateful to Angela Wade for help in preparing the figures.

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